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Dottorando: Dr. A. Cossu

Azathioprine-Induced Peripheral T Cell Apoptosis And Drug Response In Patients With Crohn's Disease

Relatore e *Tutor*

Prof. E. Corazziari

Relatore esterno

Dr.ssa M. Boirivant

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ABSTRACT

Background and Aim: the long time interval for a trial of thioupurine therapy and the potential side effects in spite of the proven efficacy, do not encourage their use as early therapeutic option in Crohn's Disease (CD). The development of tests predictive of responsiveness represents a major attempt in the clinical management of CD patients. Azathioprine (AZA) is able to induce apoptosis of T cells; therefore we analyzed the "in vitro" thiopurine-induced T cells apoptosis in a group of CD patients with known response to a previous treatment with AZA. Methods: peripheral CD4+ T cells from 16 CD patients were stimulated with αCD3/28 mAbs in the presence or absence of AZA or 6-MP or 6-thioguanine; apoptosis was assessed using Annexin V staining. Results: Apoptosis stimulation index (% of apoptotic cells in the presence of thiopurine / % of apoptotic cells in their absence) was significantly lower in non responder when compared to responder patients (1.46 (0.97-1.8) vs. 2.19 (1.58-2.65) median (range), respectively; p=0.002 by Mann Whitney test). Conclusions: evaluation of apoptosis stimulation index of peripheral CD4+T cell induced by AZA might represent a parameter useful for a proper selection of CD patients candidate to thiopurine treatment.

INTRODUCTION

Azathioprine (AZA) and mercaptopurine (MP) are established immunomodulatory drugs successfully used as chemotherapeutic in inflammatory bowel disease (IBD) (1). AZA and MP have proven efficacy in induction of remission in active CD and the response increases after 17 weeks, suggesting there is a minimum length of time for trial of thioupurine therapy (2). These drugs have a proven steroid-sparing effect (2) and efficacy in CD patients with perianal disease (3). AZA and MP are more effective than placebo for maintenance of remission in CD (4) and more recently showed efficacy in preventing CD recurrence after surgery, especially in high risk patients (5). In IBD patients treated with thiopurines, up to 20% had to discontinue treatment due to adverse events related to dose-dependent pharmacologically explainable events and dose-independent, hypersensitivity reactions (6). Therefore, although AZA and MP show proven efficacy, the relatively long term treatment required to obtain a full pharmacological effect together with possible adverse reactions, do not encourage the use of thioupurines as an early therapeutic option. The possibility to predict their efficacy in each single patient might encourage a more aggressive strategy with an early use of these immunomodulatory drugs rather then a conventional therapeutic approach (7, 8). The monitoring of 6-thioguanine nucleotides (6-TGNs) and 6-methylmercaptopurine (6-MMP) metabolites has been proposed for the evaluation of clinical efficacy (9), although a recent meta-analysis of such studies shows a relatively low sensitivity in predicting clinical response (10). Recently, it has been demonstrated that the immunosuppressive effects of azathioprine is related to its ability to induce apoptosis (11) of T cells which, in CD, is defective (12). Consequently, more recently, the evaluation of 6-thioguanosine triphosphate levels directly responsible for the proapoptotic effect and its precursor 6-thioguanoside diphosphate has been proposed for the prediction of azathioprine responsiveness in IBD patients (13*).* However, the monitoring of metabolites implies the assumption of the drug for an adequate length of time and, therefore, the exposure to the risk of adverse effects.

Aim of the present study was to analyse the “in vitro” thiopurine-induced peripheral T cells apoptosis in a group of CD patients with known response to a previous treatment with azathioprine. The effect of thiopurine on T cell apoptosis “in vitro” was studied and the results analyzed according to the response to the previous treatment.

METHODS

Patients

Adult CD patients were retrospectively selected on the basis of clinical records of all CD patients under regular follow up at the GI Unit of the University of Tor Vergata of Rome (Italy). Inclusion criteria were adult CD patients currently in follow up previously treated with AZA with clinical indication and dosage according to current guidelines (14). Patients were excluded if reliable data on clinical response could not be ascertained, or if a stable AZA therapy over a period of at least 6 months could not be recorded. AZA responsiveness was defined as steroid-free clinical remission and/or at least 50% fistulas closure for 1 month.

Blood sampling

Heparinised peripheral blood samples (20cc) were obtained from 16 compliant CD patients during a scheduled follow up visit. At the time of blood collection, clinical characteristics of patients (CD localization, disease activity, drug treatments, and time length of thiopurine-free therapy) were recorded. Blood samples were processed according to the below reported protocol. Investigators were blind to the patients clinical data during all the experimental procedure.

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque (Pharmacia Biotech, Upsala, Sweden) density gradient. Purified CD4+T cells were obtained from PBMC by immunomagnetic negative selection using CD4+ T cell isolation Kit II (Miltenyi Biotec, Auburn,CA, USA) The resulting (unbound) CD4+T cell population contained greater than 96% CD4+ cells as assessed by flow cytometric analysis.

Cell cultures

CD4+T cells (1 x 10^6) were resuspended in 1 ml of complete medium (RPMI 1640 supplemented with 2mM *L*-glutamine, 100U/ml of penicillin, 50 μg/ml of streptomycin, 25 μg/ml of gentamycin and 10% heat-inactivated FCS) and cultured for 4-5 days in 24-well plates in a humidified 5% CO2 incubator in the presence of recombinant IL-2 (20 U/ml; Roche Diagnostic, Indianapolis, IN, USA). Cells were cultured either in the absence of any stimulus or in the presence of plate-bound anti-CD3 Ab (0.04 μg/ml; BD Biosciences Pharmingen, San Diego, CA, USA) and soluble anti-CD28 (1 μg/ml; BD Biosciences Pharmingen), in the presence or absence of AZA, 6-MP or 6-thioguanine (6-TG) (all from Sigma-Aldrich) used at 5 μM concentration, for 4-5 days. In few experiments, in additional cultures, cells were incubated with AZA 10μM. At the end of the culture period, supernatants were collected and stored at -20°C for further analysis and cells were collected for apoptosis evaluation. Apoptosis was assessed using Annexin V staining (BD Biosciences

Pharmingen, San Diego, CA, USA) and analyzed by FACScan cytofluorimeter and expressed as % apoptotic cells. The degree of apoptosis induction was expressed as apoptosis stimulation index using the following formula: % apoptosis in culture in the presence of AZA/% of apoptosis in the absence of AZA

Cytokine measurement

The amount of IFN-γ in cell supernatants was measured by ELISA using commercially available kits (BD Biosciences Pharmingen, San Diego, California, USA) following manufacturer's instructions.

Statistical evaluation

Mann-Whitney and Wilcoxon test were used as appropriated

RESULTS

Patients

The clinical characteristics of patients included in the study, as well as the indication for AZA treatment and response evaluation are summarized in Table 1. All patients received AZA (2 mg/Kg) for at least 6 months. Response to AZA was observed in 9 out of the 16 patients.

Apoptosis evaluation

In initial experiments we evaluated the apoptosis induced by AZA, 6-MP and 6TG treatment on unstimulated and αCD3/CD28 stimulated CD4+ T cells. As expected (11), unstimulated cells co-incubated with the drugs showed level of apoptosis comparable to that observed in unstimulated untreated cells (data not shown), furthermore no difference was observed among AZA, 6-MP and 6TG. As opposite, AZA was able to increase the % of apoptotic cells observable in CD4+T cells stimulated with aCD3/28 mAb and the apoptosis induction by AZA, 6-MP and 6TG was comparable (data not shown).

Fig. 1A shows the % of apoptosis observable in αCD3/aCD28 stimulated T cells in the presence (treated cells) or absence (untreated) of AZA in CD patients according to the response to AZA. The % of apoptosis in stimulated but untreated cells was not different between responder (R) patients and not responder patients (NR) (R: 20.2 (10.8-38.8); NR: 23.8 (8.1-58.3) median (range) p=0.60 by Mann Whitney test). All the patients showed an increase in the % of apoptotic cells in the presence of AZA ($p<0.05$ by Wilcoxon test). However, αCD3/28 stimulation in the presence of AZA was associated with higher (albeit not statistically significant) % of apoptotic cells in responder patients when compared to non responders (R: 44.3 %(23.6-89.1); NR: 35.6% (15.2-96.1), median (range).p=0.25 by Mann Whitney test). Comparable results were obtained with αCD3/28 stimulation in the presence of 6-MP and 6TG (data not shown).

To quantify the ability of AZA to increase the apoptosis observable after aCD3/aCD28 stimulation, we conveyed the data as apoptosis stimulation index. As shown in Fig. 1B NR patients showed a significant reduced apoptosis stimulation index when compared to R patients. In two NR patients apoptosis was evaluated after incubation of lymphocytes with AZA 10μM also. In both cases the % of apoptosis observed was not different from the one observed in cultures containing AZA 5μM (data not shown)

IFN-γ production:

We next evaluated the amount of IFN-γ in the supernatants of cell cultures utilized for apoptosis evaluation. As shown in Fig. 1C and D, incubation with AZA reduced the IFN-γ production in R patients to a greater extent when compared to NR patients. Of interest, NR patients showed a significant increase of basal IFN-γ production by stimulated CD4+T cells when compared to R patients (NR: 209518 (2800 -2.1020e+006); R: 12668 (1700 -160076) pg/ml median (range) p=0.04 by Mann Whitney test) .

DISCUSSION

In the present study we describe the differential ability of AZA to induce apoptosis of aCD3/28 stimulated peripheral blood CD4+T cells in patients with a previous response to AZA when compared to patients that did not respond to a previous AZA treatment. AZA induced a significantly higher degree of apoptosis in peripheral blood cells from patients with previous response to AZA, than in cells from patients who did not respond to a previous AZA treatment. The observed different apoptosis response to AZA was not related to a different % of apoptosis observable between the two groups of patients after aCD3/28 stimulation and, therefore it seems the expression of an intrinsic difference in the response to AZA.

Factors that may influence the response to thiopurines include the thiopurine methyltransferase (TPMT) activity which influences the active 6-thioguanine nucleotides (6-TGMP, 6-TGDP, and 6-TGTP) levels. TPMT activity shows wide interindividual differences because of common genetic polymorphisms. However a very high TPMT activity that is associated with pharmacologic resistance to AZA has been described in 1% of IBD patients while around 90% present a normal TPMT activity (15). Moreover, we observed similar differences in the induction of apoptosis between responders and non responders patients when CD4+T cells were stimulated in the presence of 6-TG which is less affected by TPMT (16). Therefore, it is unlikely that interindividual differences in TPMT activity might account for the difference in apoptosis observed in the present study. Factors like disease activity and treatment at the time of blood collection might have influenced the "in vitro" response to azathioprine observed in the present study. However, disease activity as well as treatment with AZA was comparable between the groups of responder and not responder patients at the time of blood sample collection. The majority of non responder patients were, in fact, treated with systemic or local steroids and/or infliximab. In spite of the known ability of steroids (17) and infliximab to induce apoptosis (18), we did not observe differences in the % of apoptotic peripheral CD4+T cells after aCD3/28 stimulation in the

group of non responder when compared to responder patients. On the basis of this observation it seems unlikely that treatment at the time of blood collection might have influenced the apoptotic response to AZA in our study.

As a consequence of the differential apoptosis observed between responder and non responder patients, we observed a greater reduction of IFN-γ content in cultures from responder when compared to non responder patients. Interestingly, a significant higher levels of IFN-γ was observed in CD4+T cells stimulated with aCD3/28 in non responder patients when compared to responders. Since no difference in the % of apoptosis was observed between non responders and responders upon stimulation with aCD3/28, this condition might specifically influence the apoptotic response to AZA. This observation, together with the observed significant increase of the disease duration in non responder patients, when compared to responders, suggests that the response to AZA might be influenced by qualitative/quantitative differences in the inflammatory milieu somehow associated with the duration of inflammation. Whether IFN-γ production might by itself influence the "in vitro" apoptotic response to AZA or represent a marker for different factors able to influence the apoptosis process remains to be investigated.

In summary, we observed a differential "in vitro" thiopurine-apoptotic response of peripheral blood CD4+T cells isolated from CD patients responders or non responders to AZA. This feature seems to be independent from the time interval between AZA treatment and blood sampling and rather linked with an individual trait of the inflammation. Once confirmed in a large prospective study, present findings suggest that the evaluation of apoptosis stimulation index of peripheral CD4+T cell after incubation with AZA might represent a parameter useful for a proper selection of CD patients candidate to thiopurine treatment.

Table1. Clinical Characteristics of Crohn's Disease Patients.

	Responder Patients (n=9)	**Non responder Patients** (n=7)	**p**
M/F	4/5	3/4	
Age (y)			
mean±SD	38±15	51±14	>0.05
range	16-65	33-71	
Disease duration (y)			
mean±SD	14±9	25±8	0.02
range	3-27	12-36	
Indication for AZA treatment			
Steroid-dependence	4	4	
Steroid dependence and fistula (perianal disease)	5 (4)	3	
CD localization at the time of AZA treatment (post-surgical recurrent disease)			
Small bowel	5(3)	6(5)	
Small and large bowel	4	1	
CD localization and behavior at the time of blood collection			
Small bowel (recurrent disease)	5(3)	6(5)	
Small and large bowel (recurrent disease)	4	1	
Fistulae (perianal disease)	2 (1*)	3	
CD activity at the time of blood collection			
Active (CDAI >150)	2	2	
Remission (CDAI ≤150)	7	5	
Treatment at the time of blood collection			
AZA	3	2	
5-ASA	8	6	
Prednisone		2	
Budesonide	2(1*;1§)	3	
Infliximab**		3	
Duration of AZA treatment at the time of blood collection			
(months)	14, 12, 42	11, 40	
AZA-free period at the time of blood collection			
(months)	84*, 14§,41, 2, 12,12	18, 3, 6, 5, 3	

* One steroid-dependent patient with perianal fistula showing AZA response with steroid-free remission and fistula closure, voluntary discontinued AZA and experienced recurrence of perianal fistula and steroid-dependence.
§ One steroid-dependent patient who voluntary discontinued AZA showed recurrence of steroid-dependence.
** Maintenance treatment.

Fig. 1

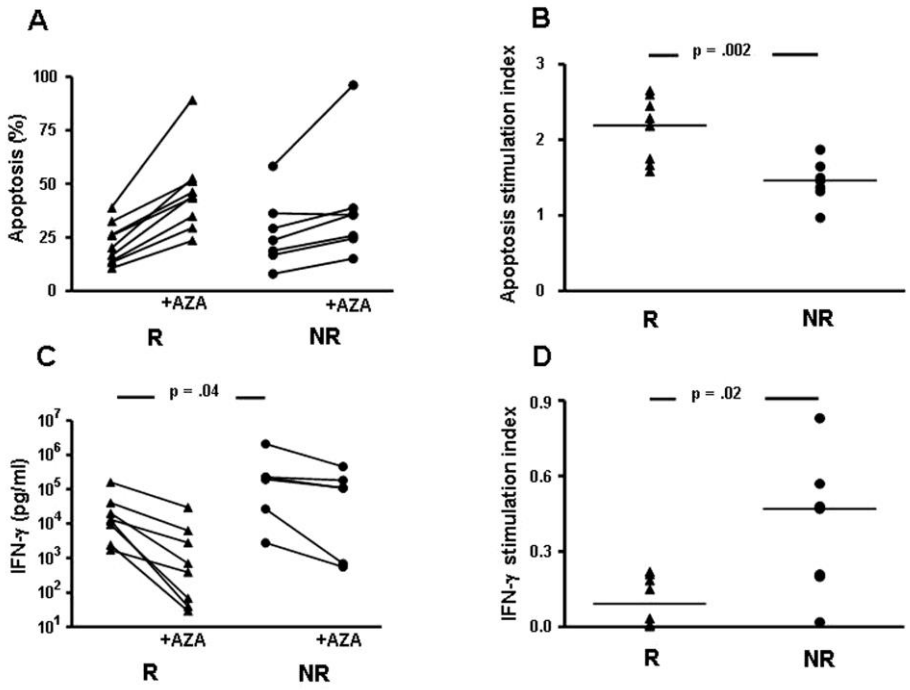

Figure legend

Fig. 1. "In vitro" effect of AZA on peripheral blood CD4+T lymphocytes in patients responder or non responder to AZA treatment (2 mg/Kg). Cells were stimulated with aCD3/28 mAbs in the presence or in the absence of AZA (see methods). Panel A: % of apoptotic cells in cell culture of CD4+T cells in the absence or in the presence of AZA (+AZA); Panel B: apoptosis stimulation index. Panel C: IFN-γ production in cell culture of CD4+T cells in the absence or in the presence of AZA (+AZA). Panel D: IFN-γ stimulation index. R= responder patients, NR= non responder patients.

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